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We heart cultured hearts. A comparative review of methodologies for targeted differentiation and maintenance of cardiomyocytes derived from pluripotent and multipotent stem cells

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Abstract

Human cell lines, including disease cell lines are often superior to routine animal models for the purposes of rapid and safe assessment of the effects of different agents that may modulate myocardial functioning under physiological and pathological conditions. There are several currently existing methodologies for derivation of cardiomyocyte-like cells by targeted differentiation from pluripotent cells and by reprogramming/ transdifferentiation from other types of cells (multipotent progenitors, somatic cells, etc). The present paper reviews the potential sources of cells capable of differentiation along the cardiomyocyte lineage; the existing methodologies for targeted differentiation, outlining the specificities of each method; and the markers for differentiation along the mesodermal and the cardiogenic lineage. The yield of robustly beating cells expressing cardio-specific proteins derived by any of the existing methods, however, still rarely exceeds 70-90 %, even with the newly developed approaches for increasing the differentiation capacity. There still is significant variance in the results obtained by different research groups and even between different experiments carried out in the same laboratory, with the same type of cells and same general type of protocol. Derivation of new lines of human pluripotent and multipotent stem cells according to standardised protocols and in completely defined; xeno-free conditions may increase the reliability and reproducibility of research and speed up the development of potential clinical applications.

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Abbreviations: **APC** – Adenomatous polyposis coli; **APEL** – Albumin, polyvinyl alcohol, essential lipids; **bFGF** – Basic fibroblast growth factor; **BMP** – Bone morphogenetic protein; **CPC** – Cardiac progenitor cells; **DKK1** – Dickkopf homolog 1; **DMSO** – Dimethylsulfoxide; **ESC** – Embryonic stem cells; **hESC** – Human embryonic stem cells; **mESC** – Murine embryonic stem cells; **GMP** – Good manufacturing practice; **GSK3β** – Glycogen synthase kinase 3 isoform beta; **iPSC** – Induced pluripotent stem cells; **LIF** – Leukemia inhibitory factor; **MSC** – Mesenchymal stem cells; **N-CAM** – Neural cell adhesion molecule; **PDGFR** – Platelet-derived growth factor receptor; **shRNA** – Small hairpin RNA; **SIRPA** – Signal regulatory protein1, alpha type; **SSEA** – Stage-specific antigen; **TCF/LEF** – Transcription factor F/ lymphoid enhancer factor; **TGF-β** - Transforming growth factor β; **VCAM1** - Vascular cells adhesion molecule 1; **VEGF** – Vascular endothelial growth factor; **VEGFR** – Vascular endothelial growth factor receptor; **VSC** – Vascular stem cells; **WHO** – World Health Organisation; **Wnt** – Wingless/INT ligand

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1. The potential of in vitro cell culturing for providing standardised, reliable and safe cardiomyocyte lines for the purposes of research and potential clinical applications

Cardiovascular disease is responsible for a significant proportion of morbidity and mortality in industrialised countries. The risk for development of heart disease increases steadily from a couple per cent at the age of 20 to over 20 % in people aged >65 years. According to data obtained from the WHO official webpage, cardiovascular disease is the most common cause of death among adults on a global scale (48 %), exceeding the percentages of deaths related to cancer, diabetes and chronic lung disease [*WHO global mortality data*]. It could only be expected that the prevalence of cardiovascular disease would continue to grow in the near future, as the average human lifespan has increased with 25-30 % in the last 100 years. Massive effort is invested worldwide into prevention of cardiovascular disease (identification of risk factors, minimisation of their impact, early detection); treatment, when the disease has already developed; prevention of complications; and increasing quality of life for the patients. Modern cardiology has at its disposal a variety of therapeutic options combining conservative treatment and surgery to achieve optimal results for the patient. Pharmaceutical science provides many newer and potentially more effective agents every year. New medicinal products, however, must undergo prolonged and rigorous testing before they reach potential patients, as the safety concerns associated with their use may outweigh the potential benefits. This happened with cerivastatin, marketed as a potent cholesterol-lowering drug from the 90-ties of the XX century and withdrawn from the European market in 2001 because of risks for severe rhabdomyolysis; and troglitazone (an antidiabetic drug, approved for sale on the British market in July 1997 and withdrawn three months later because of increased risk for severe hepatotoxicity).

Over the history of medicine, animal models have provided ample opportunities to study the physiology and the pathology of the heart and the vascular system and to try out new medicinal agents and surgical techniques. However, it may be difficult (and sometimes, even dangerous) to translate results obtained in animal models to primates and, specifically, to man. Modelling the functioning in normal and in pathological conditions of a vitally important organ, as is the heart, on primate models, may pose multiple ethical issues that are virtually impossible to overcome at present. Deliberately producing a disease phenotype in primates and humans in order to monitor the course of a disease or try out a new therapeutic approach is likely to cause needless pain and suffering and is therefore absolutely unacceptable. Cultured cardiac cells offer the opportunity for observing and analysing data about the influence of different agents on the contractile function of the heart without using living organisms as test subjects. The use of human cells to study human conditions may help to avoid at least some of the pitfalls that occur in translation of results obtained in animal models to man. Cultured cells possess great value as a model system in which to analyse the safety profile of a drug or a therapeutic regimen, or, in case of disease cell lines, the effectiveness of the particular agent in improving the functioning of the affected cells. The stimulus-response relationship is easier to monitor in cells rather than in complex living organisms and compensatory mechanisms that may suppress, mask, or augment a response may be less difficult to identify and eliminate.

Explanted cardiomyocytes (e.g. obtained by myocardial biopsies) may be maintained in specialised nutrient media. However, primary cultures of cardiomyocytes are not long-lived enough to be seriously considered for potential therapeutic uses. Freshly isolated cardiac tissue is normally viable for less than 12 hours and cultured adult cardiomyocytes may remain alive and robustly pulsating for no more than several weeks. Therefore, primary cultures of cardiac cells could not provide a steady source of cells to try out different therapeutic regimens, as their lifespan in vitro is often shorter than the length of the average therapeutic trial. Consistency in the properties of the cultured cells used for research is crucial for the establishment of reliable experimental protocols. Primary cultures of cardiomyocytes, however, may have significant culture-to-culture and batch-to-batch variance in their properties, as they originate from different organisms and are, therefore, a product of the combined action of a different genetic background and a different environment. Thus, the results may not be reproducible, although they were obtained with essentially the same cells treated with the same agents, and the analysis of the results may be complicated by a variety of unknown factors.

The developments in stem cell science over the last three decades provide the unique opportunity of having a virtually inexhaustible stock of high-quality cells (including human cell lines from healthy individuals as well as disease cell lines) to use in research and drug development. These cells are derived, cultured and induced into specific differentiation pathways according to standardised protocols and their properties are characterised by standardised sets of markers in order to minimise the interference of factors of unknown origin, including contact with products and substances of non-human origin such as animal feeder cells, animal sera and growth factors. Fig. 1 presents cardiomyocyte clusters cultured *in vitro* in xeno-free conditions in a round bottom 96-well plate.

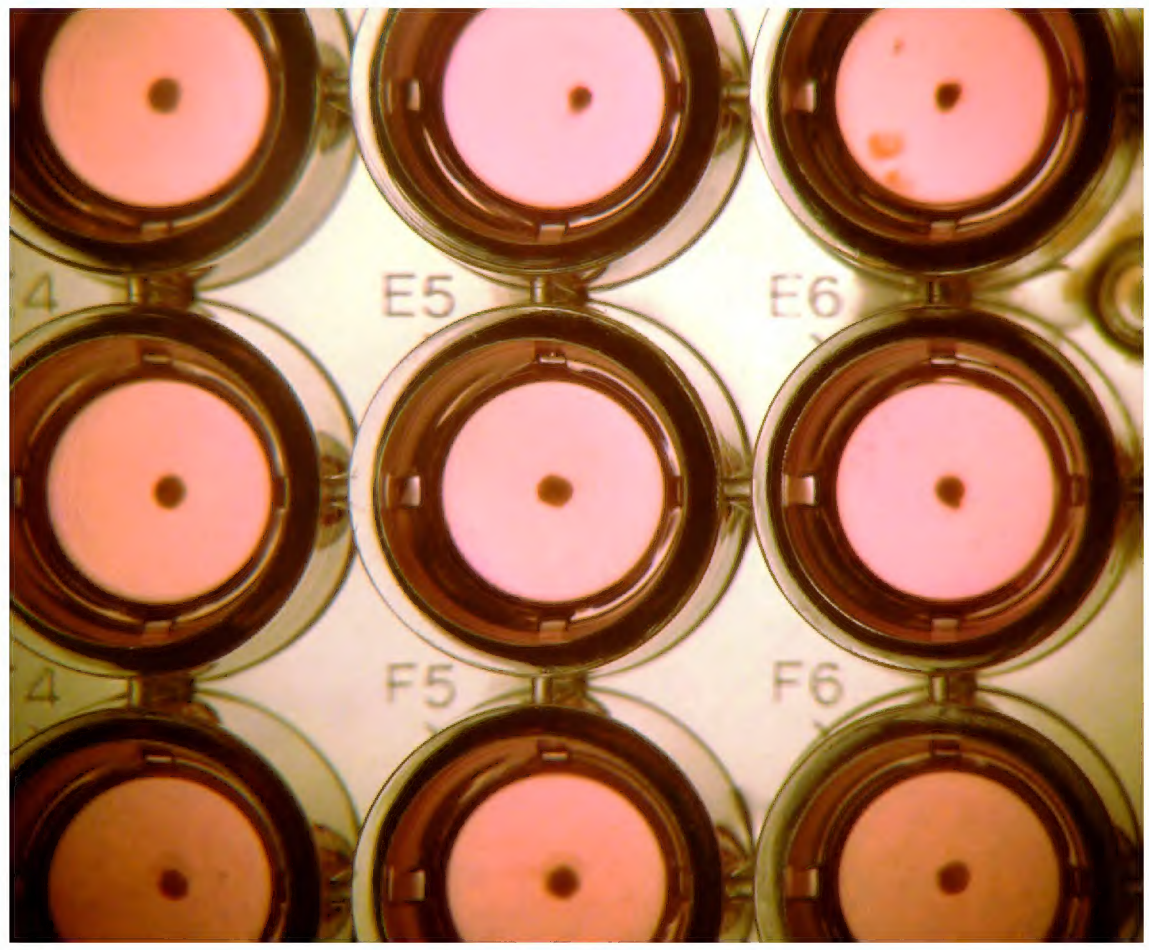

Figure 1. Cardiomyocyte clusters cultured in a round bottom 96-well plate (photograph provided by the authors).

Cultured pluripotent cell lines may be maintained in their undifferentiated state for a long time, and many batches of differentiated cells with consistent quality may be generated, maximising reliability and reproducibility of results. The use of cardiac cell lines that are derived from the same original undifferentiated cell line allows that multiple drug trials may be carried out with cells with the same basic properties, facilitating the interpretation and analysis of data. Indeed, the derivation of human embryonic stem cells (hESC) is still associated with significant ethical issues and may be restricted in some countries, but these ethical problems are on a smaller scale compared with the potential issues associated with experimentation, even with curative intent, on human beings. Fig. 2 presents a beating cardiomyocyte cluster derived *in vitro* from a human embryonic stem cell line.

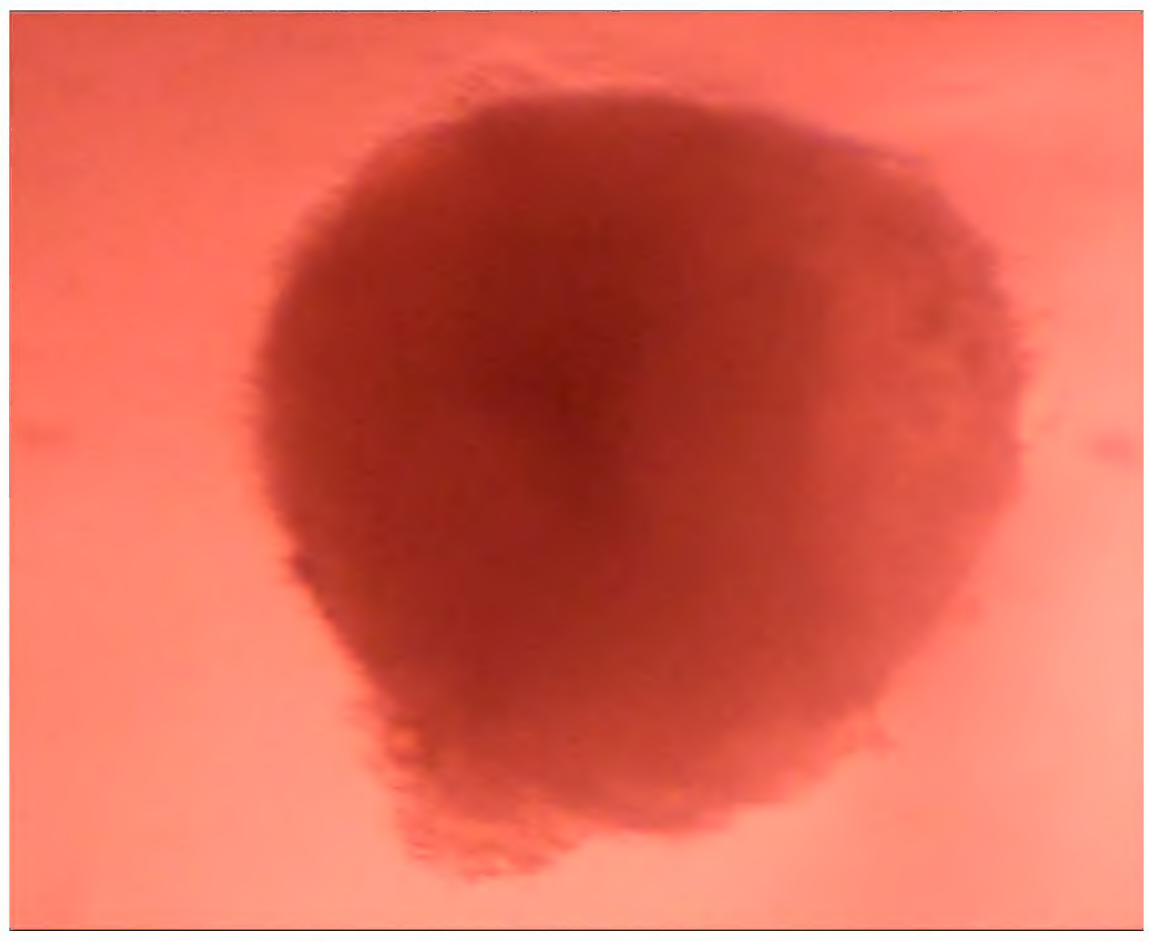

Figure 2. Spontaneously beating cardiomyocyte cluster derived in vitro from a human embryonic stem cell line (photograph provided by the authors). Characteristic rhythmical beating may be seen in supplemental videos 1 and 2. Supplemental video 1 - spontaneously beating cardiomyocyte clusters derived from hESC cell line. Beating rate of 30 beats per minute.Supplemental video 2 - spontaneously beating cardiomyocyte clusters derived in embryoid bodies from hESC cell line. Beating rate of 36 beats per minute. Videos provided by the authors.

Cardiac pacemaking, stenting, bypass surgery, valve replacement and other approaches may significantly improve the quality of life of patients with cardiovascular disease. However, there is still little to do when the function of the heart is irreversibly and severely compromised. For many decades, the main efforts in the field of care of patients with high-grade cardiac failure were targeted predominantly at improving surgical technique for heart and heart-lung transplantation, the degree of resolution of tissue compatibility typing and the establishment of larger and more representative databases for rapid and accurate donor-recipient matching. At present, transplantation of a heart taken from a deceased allogeneic donor is still the best option for patients with severe heart failure that is not treatable by any other means. However, the use of allogeneic tissues and organs of human origin are associated with specific risks. Among these risks are transmission of blood-borne infections (hepatitis viruses, human immunodeficiency virus, human T-lymphotropic virus, Epstein-Barr virus, cytomegalovirus, and the risk for immunological conflict between the transplanted tissue or organ and the recipient's immune system. Transplantation of tissues of animal origin such as porcine or bovine bioprostheses (e.g. heart valves) may also be associated with risks of transmission of zoonotic infections (e.g. the Nipah swine virus) and, in rare cases, may cause hyperacute rejection in human recipients [1].

Modern cell culturing is rapidly transitioning to completely animal product-free conditions for derivation, maintenance and differentiation of human cells, eliminating the risks for transmission of infections to potential recipients and immune reactions to proteins and other compounds of animal origin. Stable lines of human cells cultured in standardised conditions may potentially provide significant opportunities in the field of regenerative medicine. It may be possible, in the not-so-distant future, to ensure that tissues and organs that have aged or have become injured because of a disease or any other reason, may be safely replaced, or, at least, that their functioning may be supported by a fresh supply of specialised cells of the same type as the damaged cells. The risks of severe immunological conflicts may also be easier to resolve with cell lines as potential sources for cells and tissues and for transplantation than with donated tissues and organs. It is believed possible that after careful analysis of the distribution of the HLA types in the particular population, the minimal set of cell lines potentially intended to produce differentiated cells for transplantation purposes may be selected specifically so that their HLA types would match with high degree of compatibility (allowing for ≤ 2 mismatches) the HLA characteristics of a significant percentage of the population [2, 3].

Cultured cardiomyocytes obtained by differentiation of pluripotent cells (embryonic stem cells, induced pluripotent stem cells (iPSC)) or, possibly, by dedifferentiation/transdifferentiation from other cell types (e.g. mesenchymal stem cells (MSC) from umbilical cord) have a lot to offer to modern biomedical science. Indeed, at present the potential advantages are related predominantly to drug design and testing studies carried out in cultured cells than to their direct use for transplantation purposes. The concept of growing a new heart from cells in a culture dish still belongs to the future, but, nevertheless, it is a future much closer than it was in the XX century. This paper reviews the most commonly used protocols for targeted differentiation of pluripotent cells towards cardiogenic fate, outlining the mechanisms of action of different agents used in these protocols; the markers used for characterisation of the mesodermal and cardiogenic lineages; and summarising the specific points concerning the potential uses of cardiomyocytes derived *in vitro*.

2. Brief historical overview of the attempts to cultivate rhythmically beating cardiac cells under *in vitro* conditions

Initial attempts for establishment of primary cultures of human cardiac cells date from more than a century ago, with the experiments of the anatomist Montrose Burrows and the virtuoso surgeon Alexis Carrell [4, 5]. It is easy to imagine the fascination that the idea of the maintenance of a living organ outside the body held for them, as it was believed at the time that the 'freshness' of the explanted organ and the skill of the surgical team were the crucial factors for the success of a transplantation. Burrows and Carrel observed that explants from embryonic chicken hearts placed in nutrient medium would continue pulsating rhythmically for days [4, 6]. They also reported that that single cells would become detached from the bulk of the tissue, move actively away from it and grow into the nutrient medium. Carrel and Burrows noted that successful culturing of explants from embryonic chicken hearts while retaining the defining characteristics of the tissue was hardly possible beyond several weeks, and that the viability of the tissue could be significantly prolonged by replacing the nutrient medium at regular intervals [4, 6]. More than 4 decades later, Margaret Cavanaugh reported that under in vitro conditions cells from embryonic chicken heart that were completely separated from one another by means of trypsinisation were capable of continued rhythmical contraction [7]. Cavanaugh also noted that these cells could divide and migrate away from the other cells in the nutrient medium. Later, contractile activity

modifiable by cardiac drugs was observed in isolated embryonic rat heart cells *in vitro* [8] and in cells from adult rat hearts [9].

When the first lines of embryonic stem cells were created in the 80-ties (murine embryonic stem cells, mESC) [10, 11] and the late 90-ties of the XX century (human embryonic stem cells, hESC) [12], cardiomyocytes were among the first cell types that were reported to be derived from differentiation of embryonic cells *in vitro* [13]. Undoubtedly, this was related, on the one hand, to the relatively common occurrence of spontaneous differentiation along the cardiogenic lineage, and, on the other hand, to the characteristic phenotypic feature of rhythmical pulsation that facilitated the acknowledgement of the presence of cardiomyocytes in embryoid bodies and cells grown in monolayers. The protocols for targeted differentiation of embryonic stem cells to cardiomyocyte-like cells by sequential induction (that is, directing the cells to the mesodermal lineage first and then into the cardiogenic pathway) developed over the next decade years [14-17]. The yield of cardiomyocytes was, however, quite low, especially with embryoid body-based protocols that patently provided no more that several per cent of robustly beating cells. Different small-molecule approaches to increase the yield were tried and tested, and in 2013 was published a report that treatment of differentiating cells with dimethylsulfoxide (DMSO) dramatically increased the differentiation rates in all germ layer lineages, including the cardiogenic lineage [18]. Fig. 3 and 4

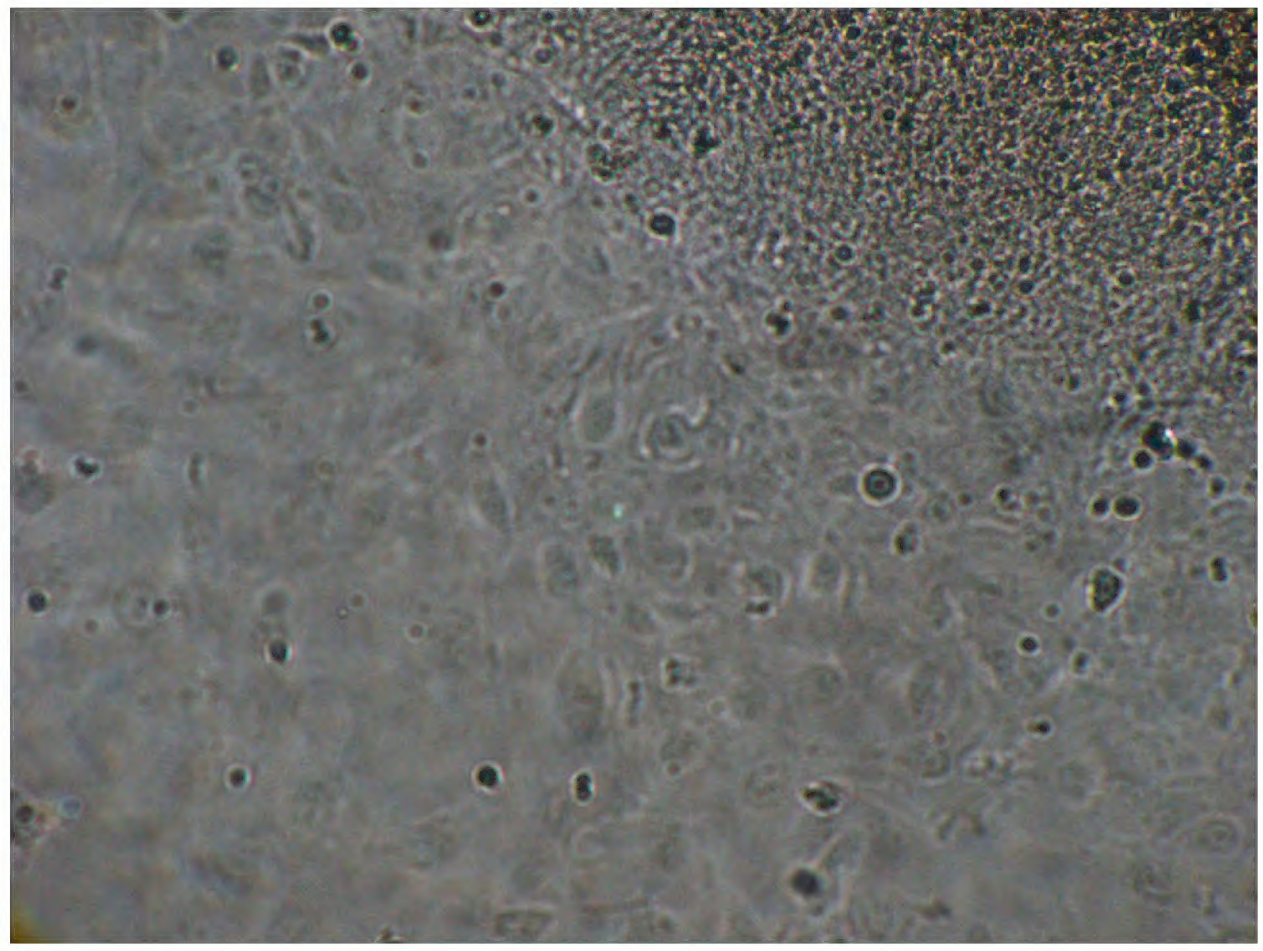

Figure 3. Cardiomyocytes growing in monolayer (photographs provided by the authors).

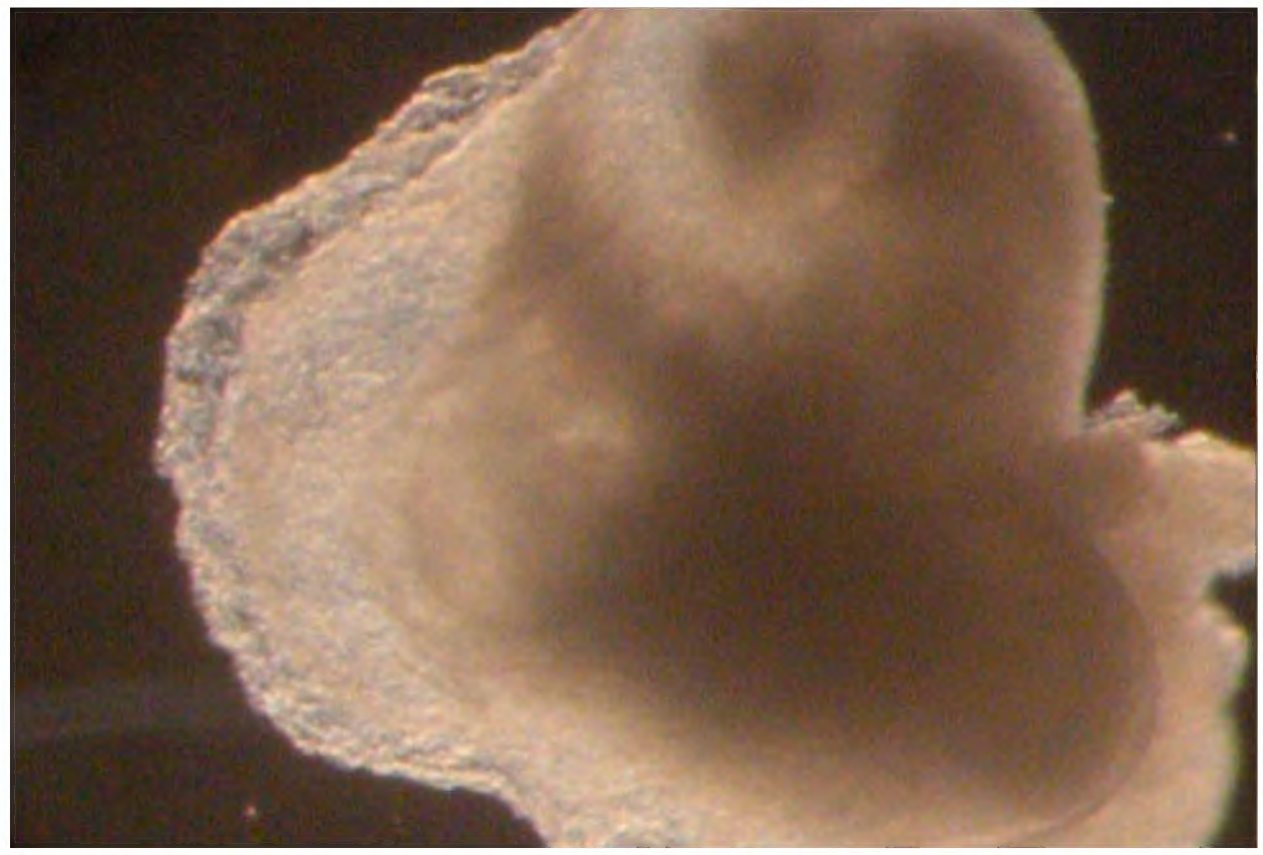

Figure 4. Cardiomyocytes grown in embryoid bodies derived from human embryonic stem cell lines (photograph provided by the authors).

present cultured cardiomyocytes (grown in monolayer and embryoid bodies, respectively) derived from human embryonic stem cell lines. Characteristic rhythmical beating may be seen in the supplemental videos.

In 2008, three independent research groups reported the derivation of cardiomyocytes from murine iPSC using embryoid body-based and monolayer-based differentiation protocols [19-21]. Later, the capacity for derivation of cardiomyocyte-like cells was demonstrated with human iPSC generated using the 4-gene system (*OCT4*, *SOX2*, *NANOG*, and *LIN28* transgenes) [15] and the three-gene system (*OCT4*, *SOX2*, and *KLF4*) plus a histone deacetylase inhibitor (valproic acid) [23].

3. Cardiac development in the human embryo

Pluripotent stem cells (embryonic stem cells and induced pluripotent stem cells) maintained in culture are commonly used for derivation of cardiomyocytes *in vitro*. The essential stages of in vitro differentiation of embryonic stem cells resembles early embryonic development, therefore, we will outline the essential points of the formation of the mammalian heart and the vascular system. The heart is the first organ to form in developing vertebrate embryos. The specification of the cardiogenic lineage occurs mainly via cell-cell interaction between the cells of the endoderm and the mesoderm [24-26]. The cardiogenic fate of embryonic mesoderm is dependent on the expression of several specific transcription factors (GATA, NKX2, MEF2A, TBX5, myocardin) [27, 28]. Signalling via Wnt/β-Catenin is crucial in the specification of the cardiogenic mesoderm [28]. The heart forms from the anterior and lateral mesoderm of the mammalian embryo, whereas the cardiac arteries arise predominantly from the dorsal mesoderm. In human embryos, the heart tube is formed in the 3rd gestational week. It is capable of rhythmical contraction in peristaltic waves by day 22-23 of pregnancy and maintains a steady blood flow in the embryo by gestation week 4-5 [reviewed in 29]. The vascular system of the heart develops in pace with the heart tube. Heart chamber septation is complete by the 8th week in the human embryo and heart valves are formed by week 10. The heart is fully functional before the end of the first trimester of human pregnancy, although cardiac cell maturation continues throughout the foetal development and into the neonatal period. Defects in cardiac and vascular development occurring in the first few weeks of pregnancy may cause early embryo loss, whereas defects occurring later (e.g. at the time when septation normally takes place) may result in congenital heart defects [reviewed in 29, 30].

4. Sources of stem cells capable of differentiation into cardiomyocytes

What comes from the heart goes to the heart.
S. T. Coleridge (1772 – 1834)

4.1. Adult cardiac progenitor cells

Mammalian cardiomyocytes were long considered to be cells that lasted a lifetime and, therefore, were supposedly never replaced when damaged. Indeed, heart muscle cells are very long-lived, but, similarly to the neurons in the CNS, they may be replaced, albeit at a slow rate. In 2004, cardiac progenitor cells (CPCs) were identified in mouse hearts as well as in atrial and ventricular human cardiac biopsy specimens [31]. CPCs grew in vitro in self-adherent clusters (cardiospheres) and expressed 'stemness' markers as well as surface markers characteristic of endothelial progenitor cells (CD31, CD34, vascular endothelial growth factor receptor 2 (VEGFR-2, KDR) and the tyrosine kinase CD117 (c-Kit) [31-33]. The levels of expression of c-KIT were found to correlate with the capacity of the CPCs to colonise damaged cardiac tissue. The potential of CPCs to renew cardiac tissue in the course of individual life was confirmed in 2009-2010, when it was shown that cardiomyocytes in adult human organisms were renewed at a rate of about 1% per year at the age of 25, decreasing to 0.45% at the age of 75 [34,35]. The process apparently continues into advanced age, as functional cardiac progenitor cells have been found in hearts of individuals aged >90 [35]. According to the calculations made by the authors, about 50% of cardiomyocytes were effectively replaced in the course of the average human lifespan.

In vitro, CPCs are capable of long-term self-renewal. They may differentiate into cardiomyocytes, vascular smooth muscle cells and endothelial cells in vitro as well *in vivo* (after transplantation into immunodeficient mice) [31, 36]. Disordered colonisation of the cell niches by the embryonic cardiac precursor cells in the course of mammalian embryonic development produces congenital cardiac defects such as tetralogy of Fallot [37, 38].

In 2011, multipotent vascular stem cells (VSCs) were identified in human coronary vessels [39]. VSCs are capable of producing neural cells, chondrocytes and mesenchymal stem cell-like cells that may subsequently differentiate into smooth muscle cells [40]. The authors of the latter study propose that the activity of VSCs may be chiefly responsible for the vascular remodelling and intimal hyperplasia typical of vascular disease, as they proliferate in response to vascular injury.

4.2. Cardiomyocytes produced by differentiation of multipotent adult progenitor cells other than cardiac progenitors

Some types of multipotent stem cells that usually do not produce cardiomyocyte-like cells may differentiate into

cells with a cardiac phenotype. Spontaneous differentiation may occur after the cells have been transplanted in an environment providing the signals necessary for their routing towards the cardiogenic fate. Alternatively, the differentiation into the cardiogenic lineage may be guided, by supplementation with different growth factors or small molecules or by modulation of the expression of specific genes. Capacity for successful differentiation into cardiomyocytes has been demonstrated for several types of adult multipotent stem cells other than cardiac progenitor cells. Mostly, these were mesenchymal stem cells derived from umbilical cord, amniotic fluid, placenta or bone marrow [41-43]. In 2004 Xu et al. reported that stromal cells from bone marrow may produce cardiomyocyte-like cells when co-cultured with myocytes [44]. Stimulation of non-canonical Wnt signaling was later shown to induce cardiomyogenic differentiation in mononuclear cells from bone marrow [45]. In 2013 was reported that cells isolated from murine amniotic fluid may be induced to differentiate to cardiomyocytes [46]. They expressed markers typical of cardiac and vascular lineage, but were not fully functional cardiomyocytes. Cardiomyocyte precursor-like cells have also been successfully derived from endometrial stem cells from menstrual blood [47]. According to the authors, when transplanted intracardially in nude rats with injured myocardium, these cells were capable of restoring the cardiac function to a significant degree.

Induced overexpression of the transcription factor myocardin was reported to result in partial transdifferentiation of mesenchymal stem cells derived from human pluripotent cells into cardiomyocytes [48].

Inhibition of the expression of certain genes may also promote the transdifferentiation of bone marrow mesenchymal stem cells to the cardiomyocyte lineage. Knockdown of the expression of histone deacetylase 1 (*Hdac1*) in rat mesenchymal stem cells from bone marrow increased the levels of expression of cardiomyocyte-related genes [49]. It has been proposed that the inhibition of histone deacetylase activity downregulates the expression of genes characteristic for the undifferentiated state but upregulates differentiation-associated genes, thus accelerating the onset of differentiation [50]. One of the characteristics of the undifferentiated state is chromatin hyperplasticity, warranting the access of the nuclear transactions machinery to DNA. Histone acetylation promotes 'open' chromatin conformation whereas histone deacetylation is associated with chromatin condensation [reviewed in 51]. It is not surprising, therefore, that agents enhancing the relaxation of the chromatin state promotes differentiation, including differentiation into unusual cell types. Differentiation into the cardiomyocyte lineage of multipotent progenitors that do not normally produce cardiomyocyte-like cells, however, is rarely complete, typically with only a subset of the cardiac lineage-specific genes activated. The cardiomyocyte-like cells may express cardiac-specific markers but tend to retain essential features of their original phenotype. Indeed, bone marrow-derived mesenchymal stromal cells induced into differentiation along the cardiomyocyte lineage expressed markers of cardiomyocyte lineage (e.g. collagen type IV, vimentin, troponin T, troponin I, alpha-actinin, connexin 43, and others) but retained their stromal phenotype and did not generate action potentials or ionic currents typical of cardiomyocytes [52].

Various factors other than starting cell type may affect the outcomes of differentiation of multipotent stem cells; such are the composition of the nutrient media and the timing of the addition of supplements. For some cell types, including cardiomyocytes; mechanical stimulation may constitute a significant factor for the outcomes of differentiation. In stromal stem cells from rat bone marrow differentiated in conditions simulating increased gravity, the predominant types of specialised cells were cells that were normally subjected to sustained tensile strain (cardiomyocytes, osteocytes), whereas cells cultured in low gravity differentiated predominantly into adipocytes [53]. Similar results have been obtained with mesenchymal stem cells [54]. Recently, it was shown that placing human CPCs in an electric field stimulated the expression of genes characteristic of the cardiomyocyte lineage (GATA-4; MEF2A and other cardio-specific protein markers) [55].

The capacity of certain types of multipotent stem cells to differentiate into cardiomyocytes in vivo has been studied intensively in recent years, albeit predominantly in animal models. Allogeneic mesenchymal stem cells have been transplanted in ischemic or infarcted myocardium of mice and pigs in the hope of improving the functioning of the heart [56-58]. The results were encouraging, as pigs with transplanted cells exhibited improved cardiac neo-angiogenesis. Transplantations of cardiomyocytes derived in vitro from mESC into hearts of non-human primate (macaque) models of myocardial infarction were reported to result in apparent remuscularisation of the infarcted region [59]. The transplanted cells were perfused by the vascular system of the recipient and exhibited Ca^{2+} transients synchronised to the recipient's ECG.

Beneficial effects from transplantation of mononuclear cells from bone marrow in human patients with acute myocardial infarction have been reported in the specialised literature. The improvements were in terms of enhanced cardiac contractility and perfusion of the infarcted region; decreased myocardial inflammation and cardiomyocyte apoptosis and stimulation of neoangiogenesis [60]. Mononuclear stem cells from human cord blood and mesenchymal cells from placenta have been applied intravenously in two critically human patients with dilated

cardiomyopathy [42, 61]. According to the authors, the patients with transplanted cells showed apparent clinical improvement in the short-term.

Recently were published the results of a large double-blind study about the effects of intracardial injection of haematopoietic progenitor cells from bone marrow in perioperative settings in patients that have undergone cardiac bypass surgery [62]. The authors reported that the survival rates were not significantly different in the patients that have received the intracardial injection compared to the control group and that the cardiac function of the recipients was not significantly improved, albeit a reduction in the size of the myocardial scar was noted. It is now believed that the transplanted cells do not take over the functions of the cells that were lost due to ischemia, but, rather, that they support the recipient's surviving cardiac muscle cells by paracrine signalling, stimulating the angiogenesis in the affected area and reducing the size of myocardial lesion. Indeed, it has been demonstrated that application of medium conditioned by human mesenchymal stem cells (not the cells) also improved the cardiac function in a pig model of myocardial infarction [63].

4.3. Cardiomyocytes derived by targeted differentiation of pluripotent stem cells

4.3.1. Cardiomyocytes derived by differentiation of ESC and iPSC

The first successful attempt for derivation of cardiomyocytes from human embryonic stem cells (the H9 line, one of the commonly used in research human ES cell lines) occurred by spontaneous differentiation in embryoid bodies [13]. In 2004, cardiomyocytes derived from human ESC were shown to be capable of restoring normal heart rhythm when transplanted intracardially in pigs with complete atrioventricular block [64]. In 2007, regeneration of infarcted rat myocardium in vivo by human cardiomyocytes derived from hESC was reported [65]. The cells were committed to the cardiogenic lineage by exposure to BMP2 and SU5402 (a potent FGFR and VEGFR inhibitor). Expression of mesodermal (TBX6, FOXH1) and cardiogenic progenitor-specific genes (ISL1, NKX2.5, MEF2C, alpha-actin) was noted at day 5 of differentiation. Two months after the transplantation into immunocompromised rats with myocardial injury, the cardiomyocytes were stably integrated into the host myocardium and expressed markers typical of cardiomyocyte lineage (e.g. beta-myosin) [65]. The authors of this study claimed that teratomas were not observed in their experimental animals. Also in 2007, an independent research group demonstrated that hESC-derived cardiomyocytes transplanted in infarcted rat hearts enhanced the functioning of the damaged cardiac muscle [14]. Later, in a guinea-pig model of myocardial infarction, transplanted hESC-derived cardiomyocytes were shown to integrate rapidly with host tissue and to contract synchronously with the host myocardium [66]. In the same year, primitive cardiomyocyte precursors were derived from several different lines of human embryonic stem cells (HES3, H9 and MEL1) by targeted differentiation using a combined protocol comprised of treatment with Activin A/BMP4 followed by inhibition of Wnt signaling [67]. In 2013 human pluripotent stem cells (hPSCs) were converted to functional cardiomyocytes in a completely defined, serum-free and growth factor-free system by temporal modulation of the regulation of canonical Wnt signaling (Wnt activation via GSK3 inhibitor; followed by Wnt signalling inhibition [68].

Cardiomyocytes derived from pluripotent stem cells or reprogrammed somatic cells are capable of spontaneous rhythmic contraction and generation of action potentials and ionic currents characteristic of cardiac cells. They express cardio-specific RNA and proteins and respond to agents stimulating their contractile activity such as caffeine and ryanodine. It has been reported, however, that the 'electric phenotype' of the cardiomyocytes obtained by differentiation of pluripotent stem cells may vary, with some cells exhibiting 'sinus nodal-like', 'embryonic atrial-like' and 'embryonic ventricular-like' properties [reviewed in 69].

In 2009, cardiomyocytes and other types of specialised cells were derived from human iPSC generated by reprogramming of haematopoietic progenitors from cord blood [22, 70]. Two years later, cardiomyocyte-like cells were derived from human foreskin fibroblasts preprogrammed to pluripotency by infection with retroviral vectors carrying the human pluripotency genes *OCT4*, *SOX2*, *KLF4* and *C-MYC* (the 4-gene protocol) [71].

Cardiomyocytes derived by targeted differentiation from iPSC also exhibit the basic properties of cardiac muscle cells, namely: propensity for spontaneous rhythmic pulsation; expression of cardio-specific RNA and proteins and capacity for response to caffeine and ryanodine [71]. However, the same authors have reported that cardiomyocytes derived from iPSC exhibited negative force-frequency relationship and lower post-rest potentiation than adult cardiac cells [71]. The force-frequency relationship is termed 'positive' when contractility increases as stimulation frequencies are stepped up and 'negative' when increased frequency of stimulation is associated with reduced contraction force [72, 73]. Impaired force-frequency relationship is one of the hallmarks of the failing heart [73-75]. Post-rest contractile behaviour of myocardium is an indicator for the capacity of the sarcoplasmic reticulum for storage and release of Ca^{2+}. Diminished post-rest potentiation

of the cardiac muscle is commonly seen in various cardiovascular conditions, including progressive heart hypertrophy and dilated cardiomyopathy; as well as in normal ageing. The responses to caffeine and ryanodine of iPSC-derived cardiomyocytes were also weaker than the response of adult cardiomyocytes [71]. Caffeine normally triggers an increase in cytosolic Ca^{2+} whereas ryanodine decreases the levels of Ca^{2+}, therefore, the maintenance of the balance of Ca^{2+} levels in cardiomyocytes derived from pluripotent stem cells may not be identical to the balance in normal adult cardiomyocytes. It is also notable that while cardiomyocytes derived by targeted differentiation from embryonic stem cells were shown to alleviate arrhythmia when transplanted in injured myocardium; other authors have demonstrated that cardiomyocytes derived from hESC may possess inherent pro-arrhythmic potential [76, 77]. Non-fatal ventricular arrhythmias were observed in macaques that have received intracardially cardiomyocytes derived from differentiation of hESC [59]. The authors of the cited study specifically noted that this was not observed in previous experiments with small-animal models. It is possible that at present cardiomyocytes derived from pluripotent stem cells might be closer to modelling heart disease than normal heart function. Concerns about the safety of their potential use in clinical applications exist at present. However, cardiomyocytes derived from pluripotent stem cells seem to be an excellent model for the failing human heart, with potential applications in research of cardiac hypertrophy, cardiac arrhythmias, dilated cardiomyopathy, advanced stages of heart failure, etc.

Currently, several basic protocols for targeted differentiation of ESC and iPSC along the cardiogenic lineage have been established (for details, see below). Most of them make use, jointly or alternatively, of Activin A, bone morphogenetic proteins, Wnt signalling pathway activators, Wnt inhibitors, basic fibroblast growth factor 2 (bFGF, FGF2); vascular endothelial growth factor (VEGF), and several types of small-molecule agents such as DMSO, ascorbic acid, derivatives of vitamin D3, etc. The cardiomyocyte yield may vary from several per cent (with embryoid bodies-based protocols) to over 90 % (for monolayer cultures).

4.3.2. Cardiomyocytes derived by reprogramming of somatic cells

Functional cardiomyocytes may directly be derived from somatic cells by reprogramming. Cardiomyocyte-like cells have been generated by direct reprogramming from fibroblasts [78, reviewed in 79]. Mouse fibroblasts have been converted in vitro directly into cardiomyocytes by induction of the expression of the cardio-specific transcription factors Gata4, Mef2C, and Tbx5 [78, 80]. The reported yield of cardiomyocytes was, however, quite low [78, 81]. Later, it was shown that the induction of the expression of additional cardio-specific transcription factors, e.g. Hand2 [82], or Hand 2 and Nkx2-5 [83] increased the yield of cardiomyocytes. Delivery of cardio-specific transcription factors and/or or microRNAs to infarcted mouse hearts had also been shown to result in direct transdifferentiation of fibroblasts into cardiomyocyte-like cells in vivo and improvement of the functioning of the damaged myocardium [82, 84, 85]. Human fibroblasts have also been induced to transdifferentiate into cardiomyocyte-like cells in vitro using basically the same cardio-specific transcription factors (GATA4, HAND2, TBX5 and MEF2C) [82] or transcription factors (GATA4, HAND2, TBX5, and myocardin) plus microRNAs (miR-1 and miR-133) [86].

5. Basic types of protocols for induction of targeted differentiation along the mesodermal lineage and direction to cardiogenic fate

5.1. Nutrient media and basic supplements used for maintenance of pluripotent human cells in vitro and targeted differentiation

Targeted differentiation of pluripotent cells with the intent to produce cardiomyocytes was initally developed in spontaneously forming embryoid bodies using supplements of animal origin such as foetal serum and support substrate derived from or secreted by animal cells (e.g. gelatin, laminin or Matrigel). In its simplest version, cardiogenic induction in embryoid bodies may be carried out in DMEM medium supplemented with L-glutamine and non-essential amino acids, serum or serum replacement, beta-mercaptoethanol (an antioxidant) and bFGF, on gelatin-coated plates [14, 87]. Other variants are also available, such as co-culturing with other type of cells (e.g. mouse feeders such as irradiated mouse cells (STO cells or endoderm-like cell line, END-2); or human feeders, e.g. placenta-derived feeder cells); culturing in nutrient medium preconditioned by other type of cells (e.g. END-2 cells or mouse embryonic fibroblasts (MEF) [88-91] or by human cardiac fibroblasts [92].

Products of animal origin and/or products with undefined composition (e.g. animal serums and Matrigel) must not come into contact with cells and cell products that may potentially be used in human beings [EudraLex - The rules governing medicinal products in the European Union, vol. 4 - Good manufacturing practice (GMP) Guidelines; *http://ec.europa.eu/health/documents/eudralex/vol-4/index_en.htm*]. The methodology of dispersion of colonies of undifferentiated cells and passaging also developed in the general direction of eliminating the use of enzymatic preparations (trypsin, collagenase IV, etc.). The latter are, in virtually all cases, of non-human origin

(typically, bovine or porcine). Currently, methodologies for mechanical passaging are being actively developed [93]. Several types of specific nutrient mediums devoid of products of undefined and/or animal origin were designed specifically for differentiation of stem cells into the cardiomyocyte lineage [94, 95].

At present, TeSR1 and the related mTESR1 are among the most commonly used mediums for maintenance of pluripotent stem cells in defined, feeder-free conditions. TeSR1 and mTESR1 are composed of inorganic salts, trace minerals, growth factors, saturated and unsaturated fatty acids, amino acids, proteins, vitamins, energy sources (glucose and pyruvate) and various other constituents such as antioxidants, precursors for synthesis of nucleic acids, and pH indicators [patent No. US7442548]. TeSR1 does not contain animal serum or products of animal origin. Variations exist that are less costly but may contain products of animal origin. For example, in mTeSR1, the expensive human serum albumin is substituted by bovine serum albumin and human bFGF is substituted with cloned bFGF from zebrafish [96, 97]. TeSR2 is commonly marketed as an improved version of TeSR1, but they are essentially the same. Some authors have reported preliminary results showing that hESC that have been maintained in mTeSR1 may not readily form embryoid bodies [98], but no adverse effects on cells grown in TeSR1 as monolayers have been noted so far. Fig. 5 presents a typical colony of undifferentiated human embryonic stem cells growing in mTeSR1 on a Matrigel-coated surface.

Induction of differentiation in cultured pluripotent cells in a completely xeno-free medium usually begins with replacement of the TeSR1 medium with RPMI medium supplemented with B27. RPMI medium has several variants. Basically, it utilises a bicarbonate-based buffering system and contain the essential components listed above - inorganic salts, amino acids, vitamins, nucleotide precursors, energy sources, antioxidants, pH indicators, etc. [99]. The B27 supplement contains vitamins; hormones and growth factors; fatty acids; catalase; antioxidants and other components. It exists in two basic variants - B27 containing insulin (B27+insulin) and B27 minus insulin (B27-insulin). The presence of insulin may inhibit the differentiation towards cardiogenic mesoderm and endoderm, favouring differentiation into the neuroectodermal lineage [100]. Thus, B27-insulin is more commonly used when directing pluripotent cells to cardiogenic fate.

Embryoid body-based methods for derivation of cardiomyocytes use specialised mediums such as APEL (albumin, polyvinyl alcohol, essential lipids) and StemPro-34 [87, 95]. Growth factors need to be added to both mediums, allowing for more precise assessment of their effects. APEL medium is free of products of animal origin and may be used in embryoid body-based as well as in adherent protocols for differentiation of pluripotent stem cells. It contains recombinant human albumin, transferrin and insulin. StemPro-34 is serum-free and is commonly marketed as a medium for culturing of hematopoietic progenitors, but may be used for cardiogenic differentiation in embryoid bodies as well [101].

Monolayer protocols for induction of differentiation along the cardiogenic lineage normally use RPMI/B27 medium with Matrigel or, when completely animal-free conditions are required, synthetic products such as the

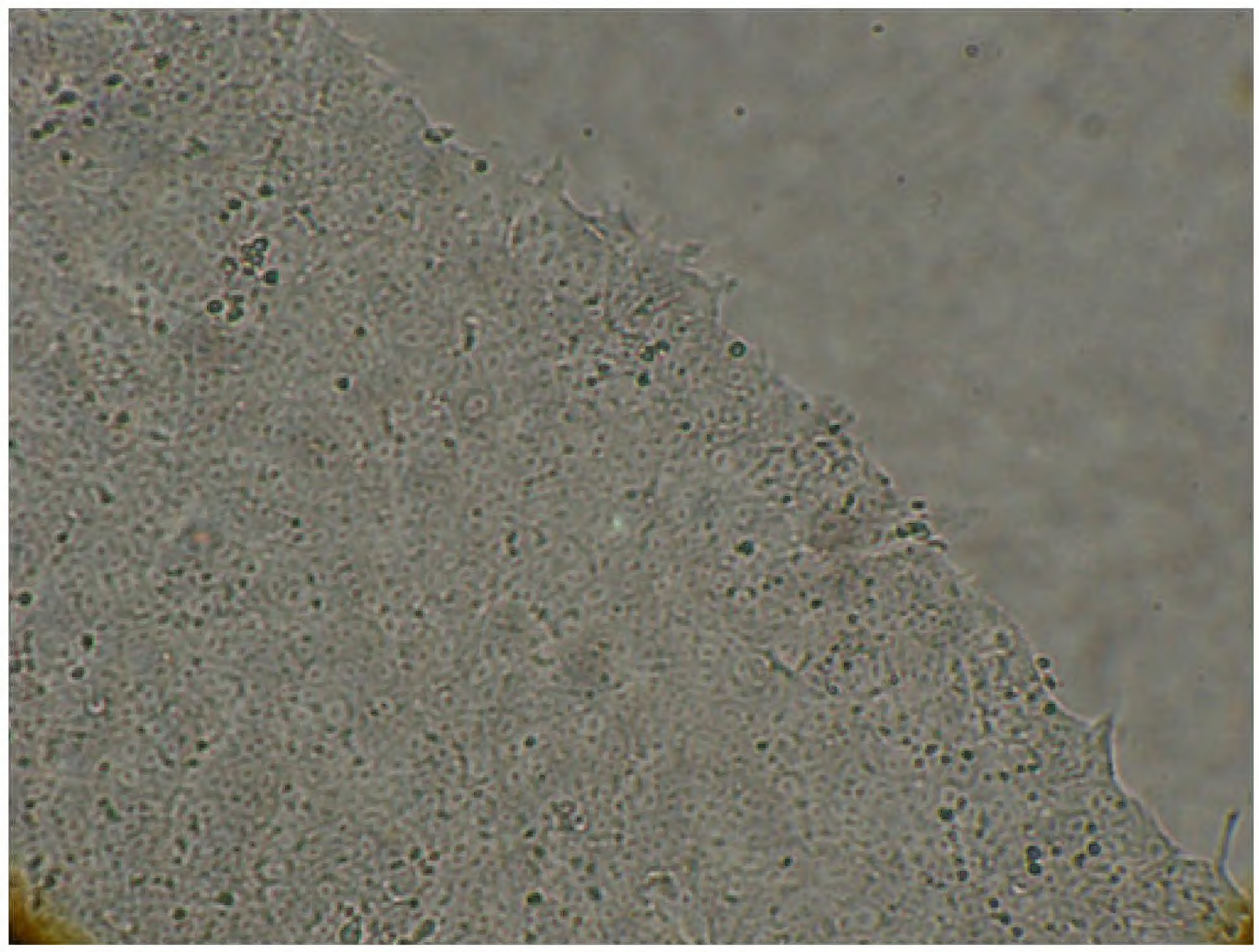

Figure 5. Undifferentiated human embryonic stem cells growing in mTeSR1 medium on a Matrigel-coated surface (photograph provided by the authors).

Synthemax surface, providing a defined solid support for cell growth [68, 98, 102].

Timed addition of specific growth factors such as bFGF and VEGF may enhance cardiomyocyte differentiation in embryoid bodies. bFGF stimulates cell division in fibroblasts in adult tissues and plays a role in would healing and tumorigenesis. It supports the growth of human embryonic stem cells in feeder-free culturing conditions and is normally included in the medium for maintenance of pluripotent stem cells in their undifferentiated state (e.g. mTeSR1) [103, 104]. In early embryos, bFGF induces differentiation along the mesodermal lineage and stimulates angiogenesis [reviewed in 105]. At post-embryonic stage bFGF controls the differentiation of cardiac precursor cells into functional cardiomyocytes [106]. Vascular endothelial growth factor (VEGF) functions in the angiogenesis in the early embryo. In vitro, VEGF stimulates the proliferation and migration of endothelial cells and plays an important role for spontaneous and damage-induced differentiation of adult cardiomyocyte progenitors into cardiomyocytes [94, 107, 108]. VEGF is typically not included in nutrient mediums and must be added manually.

5.2. Spontaneous differentiation of pluripotent stem cells along the cardiogenic lineage

Spontaneous differentiation of cultured pluripotent stem cells into beating cardiomyocytes is not unusual. A reproducible protocol for induction of spontaneous differentiation in embryoid bodies in suspension (the E9 cell line) was published first in 2001 [13]. Briefly, hESC colonies were dispersed into small clumps comprised of about half a dozen cells using collagenase IV, then allowed to grow and form embryoid bodies. Areas of spontaneous rhythmical contraction appeared by day 4th of differentiation. Spontaneous differentiation along the cardiogenic lineage was later reported to occur with other hESC lines, such as H1, H7, H9 and its derivatives (H9.1, H9.2) [109, 110] and with iPSC lines as well [22]. Fig. 6 presents cardiomyocytes produced by spontaneous differentiation in embryoid bodies from a human embryonic stem cell line.

5.3. Induction of differentiation by supplementation with Activin A/ bone morphogenetic protein 4 (BMP-4)

Differentiation into the cardiac line may be induced by more than one method. Typically, these methods are based on blocking potential pathways into differentiation in other than mesodermal lineage and limiting the potential of differentiation of mesoderm-committed cells towards endothelial and smooth muscle cells.

Activins and bone morphogenetic proteins (BMPs) are related to the transforming growth factor-β (TGF-β) family of proteins. Signalling associated with the maintenance of the undifferentiated state in mammalian ESC is transmitted via a TGF-β/Activin A/Nodal-dependent pathway [111, 112]. TGF-β and Activin A inhibit the differentiation of embryoid bodies derived from hESC to endodermal and ectodermal fates, at the same time stimulating the differentiation towards the mesodermal lineage [113]. BMPs function in various cell signalling pathways related to cell proliferation, differentiation and apoptosis [114]. Activin A is a homodimer made of two beta-A subunits of the inhibin β protein [115]. When mouse embryonic stem cells are grown in serum-free

Figure 6. Cardiomyocytes produced in embryoid bodies by spontaneous differentiation from a human embryonic stem cell line (photograph provided by the authors).

medium, BMP4 acts together with leukemia inhibitory factor (LIF) to maintain the undifferentiated state, but when LIF is withdrawn, BMP4 diverts the differentiation of mouse cells to mesodermal fate [116, 117]. In human embryonic stem cells the presence of BMP4 stimulates the differentiation of the cells towards trophectoderm or primitive endoderm [118]. When the cells have already been directed towards formation of mesoderm (e.g. by prior addition of Activin A), the presence of BMP4 drives the differentiation towards cell types originating from the mesoderm, such as the cardiogenic, endothelial and haematopoietic lineages [119, 120]. Fig. 7 presents cardiomyocytes produced in embryoid bodies by targeted differentiation using the BMP4 protocol.

The basic (4-day) protocol for induction of pluripotent human cells to differentiation along the mesodermal lineage is comprised of 24 h treatment with human recombinant Activin A followed by a 72 h treatment with BMP4 [14]. The medium (RPMI-B27, preferably minus insulin) is not changed between these two treatments. The Activin/BMP4 protocol usually results in > 30 % of beating cardiomyocytes within 2 weeks [14]. Some researchers, however, have reported significant variance in the yields obtained with cell lines and poor reproducibility of different experiments with the same cell line [14, 68, 121].

As BMP4 treatment may route differentiating cells towards non-mesodermal fates as well (e.g. hepatic and pancreatic specification), a second mesodermal induction protocol has been developed in order to decrease the yield of cells other than cardiomyocytes [122]. The cells are treated for 24 h with Activin A and Wnt3a protein together in order to enhance the formation of mesoderm [15, 16]. The protocol is carried out a single one-day phase.

Another approach that may be used for increasing the percentage of differentiating cells committed to mesodermal fate is including a pretreatment phase of activation of Wnt-associated pathways [68]. The cells are treated with a GSK3 inhibitor (for details, see below) before they are subjected to the Activin A/ BMP4 protocol. This may result in higher yields (up to >90 %) of differentiated cells with cardiomyocyte-like phenotype (cardiac troponin-positive cells) and enhance the reproducibility of results.

Inclusion of additional proteins (growth factors such as bFGF and VEGF) and Wnt inhibitors such as DKK1 (dickkopf homolog 1) may enhance mesoderm differentiation and specifically differentiation along the cardiomyocyte lineage in embryoid bodies produced by the Activin A/BMP4 protocols [94], as well as in spontaneously differentiating cells [123].

5.4. Induction of differentiation into the cardiomyocyte lineage by timed Gsk3 inhibition/Wnt signalling inhibition

Wnt (Wingless/INT) ligands play important roles in two basic signalling pathways: the canonical Wnt pathway and another two pathways collectively known as non-canonical Wnt-signalling [reviewed in 124, 125]. The canonical Wnt pathway relays signals from the cytoplasmic membrane to the nucleus via the protein β-catenin. The latter is a component of the cadherin/catenin complex. It is involved in cell-cell adhesion and transduction of signals from the cell membrane to the nucleus. Cytoplasmic β-catenin that is not bound to Wnt is continually phosphorylated by a complex made of the APC protein (adenomatous polyposis

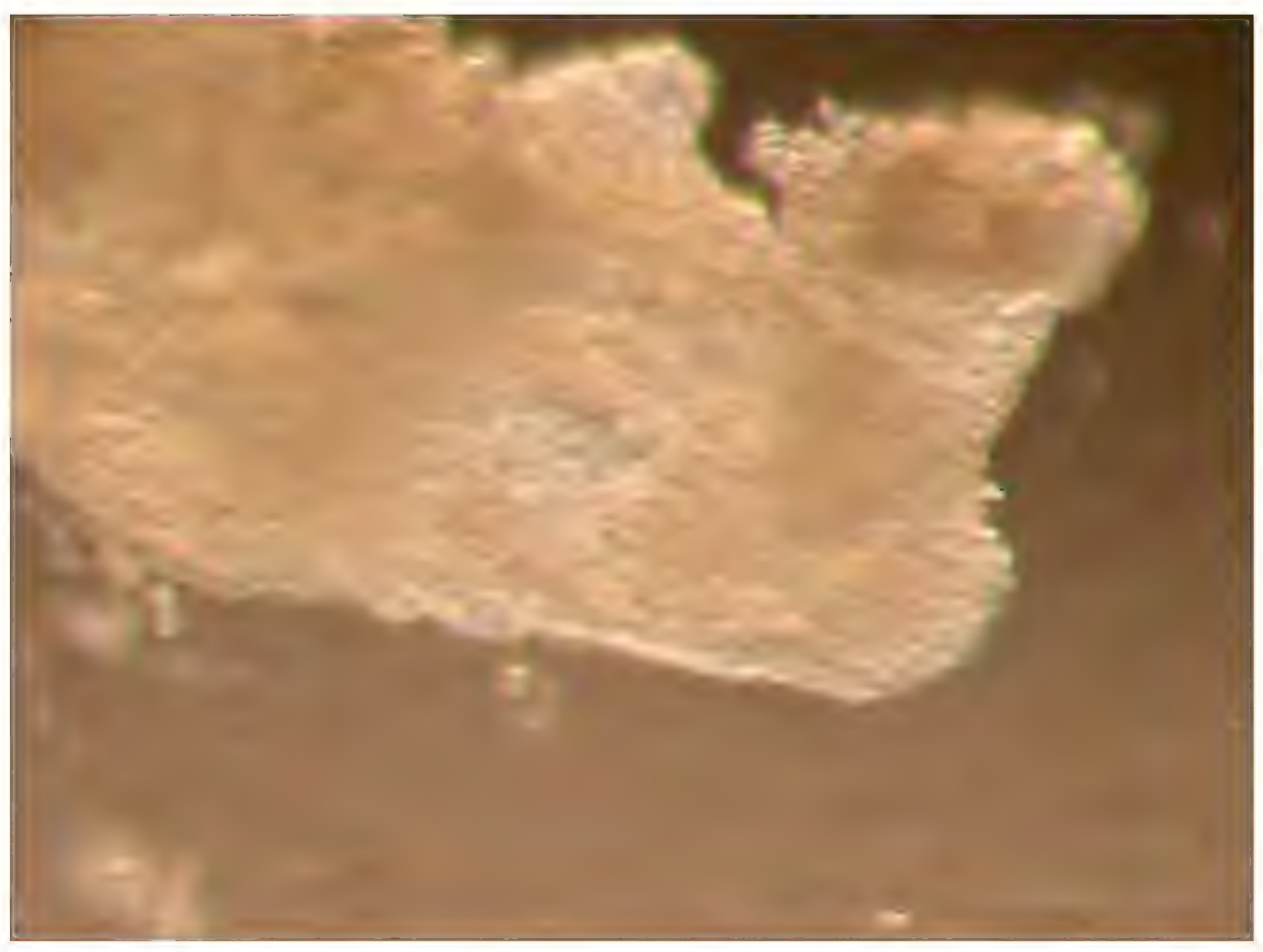

Figure 7. Cardiomyocytes produced in embryoid bodies by targeted differentiation using the BMP4 protocol (photograph provided by the authors).

coli, a tumour-suppressor), Axin (axis inhibitor 1) and the kinase GSK3β (glycogen synthase kinase 3, isoform beta). GSK3β is a multifunctional serine-threonine kinase that phosphorylates a variety of target proteins besides β-catenin such as p53, Axin, Notch, and others [126, 127]. Phosphorylated β-catenin is continually tagged for degradation via the ubiquitin-dependent pathway. Thus, in the absence of Wnt ligand, β-catenin is maintained at basal level in the cytoplasm. In the presence of Wnt ligand, a sequence of signalling events are initiated that eventually result in suppression of the degradation of β-catenin. It accumulates in the cytoplasm until a threshold level is reached, beyond which β-catenin translocates to the nucleus where it acts as a transcriptional activator of a set of downstream genes together with the TCF/LEF family of transcription factors. Nuclear TCF functions as a repressor of Wnt target genes, but in the presence of β-catenin TCF/LEF becomes a transcriptional activator of these self-same genes [reviewed in 128-130]. Among the downstream targets of TCR/LEF are the pluripotency genes *OCT4* and *NANOG*; the pro-proliferative gene *c-MYC*; the gene coding for cyclin D1, and others [125, 130, 131]. Wnt inactivates GSK3 via another signaling pathway involving protein kinase C [133, 134].

Activation of the Wnt signalling pathway in vitro (e.g. by supplementing the growth medium with GSK3β - inhibitors) normally supports the maintenance of the undifferentiated state in cultured murine and human cells [135]. Wnt signalling may play radically dissimilar roles in different stages of the differentiation of pluripotent cells, with early Wnt-associated signalling enhancing cardiomyogenesis, vasculogenesis and hematopoiesis and late Wnt signalling suppressing the differentiation towards mesodermal fate [136]. Activation of the canonical Wnt pathway is requisite for early mesoderm induction.

Modulation of the Wnt-associated signalling pathways in cultured pluripotent cells may, under specific conditions, be used successfully for induction of differentiation into the cardiogenic lineage. The timing of the treatment is critically important for the outcomes. Wnt/β-catenin signaling in human embryonic stem cells promotes differentiation to mesodermal lineages rather than self-renewal of the cells [137]. At later stages of differentiation, however, the differentiation programme for the cardiogenic lineage is characterised by inhibition of Wnt signalling. Therefore, Wnt-mediated pathways may be inhibited in a temporal fashion in order to induce mesodermal progenitors derived from pluripotent stem cells to differentiate into the cardiogenic fate [16, 138]. The inhibition may be implemented by small-molecule agents such as IWR-1, IWP-2, IWP-3, XAV939, etc., or small hairpin RNAs (shRNAs) targeting the expression of β-catenin; provided that the cells have already reached mesodermal stage. Inhibition of the Wnt-associated signalling in hESC that have been partially differentiated to mesodermal fate results in about 30 % of beating cardiomyocytes [17]. The more recently established protocol of sequential two-phase modulation of the Wnt-associated pathways has been reported to increase the yield of cardiomyocytes further [68, 102]. It is comprised of timed activation of canonical Wnt signalling followed by timed inhibition of Wnt signalling. The protocol is carried out in two phases - a pretreatment Wnt pathway activation phase, in which the cells are treated with a GSK3 inhibitor and a Wnt pathway inhibition phase [68, 139, 140]. The pretreatment Wnt signalling activation phase is carried throughout days -3 to -1 (relative to the day of start of differentiation when the maintenance medium is changed, which is day 0), and the Wnt signalling inhibition phase begins at day 1. Wnt signalling activation may be achieved in vitro with selective inhibitors of GSK3β activity, such as BIO (6-bromoindirubin-3'-oxime), or CHIR99201 [102; 135]. The subsequent Wnt signalling inhibition may be carried out using small molecule inhibitors of Wnt ligand production (KY02111, IWP agents) or shRNAs targeting β-catenin expression [102, 139, 140]. The former method (small-molecule Wnt inhibitor method) is applicable to virtually all pluripotent stem cell lines, although some authors report variable success with different cell lines [68, 140]. The latter method (using shRNAs) includes genetic modification of the cells using a viral vector and may be to be more complicated to carry out, as it includes additional steps in which the vector is amplified and inducible β-catenin knockdown clones are identified [68]. Methodologies based on sequential Wnt activation by GSK3 inhibition/Wnt signalling inactivation were reported to yield > 80 % of robustly beating cells within 12 days of differentiation [68, 102].

5.5. Increasing the propensity for differentiation along multiple lineages (including the cardiomyocyte lineage) by DMSO or other small-molecule agents

It has been repeatedly shown since the late 70-ties of the XX century that the presence of small amounts of DMSO (1-2 %) in the culturing medium of undifferentiated cells may induce cell differentiation. Initially, this was demonstrated with cancer cells (myeloid and lymphoid cell lines). Addition of DMSO induced reversible G1 phase arrest in human promyelocytic leukemia cell lines and B- and T-lymphoid cells [141-144]. Later, it was shown that murine and human stem cell lines were also induced into differentiation by DMSO [145, 146].

Treatment with 1-2 % DMSO for 24-28 h was shown to increase the yield of differentiated cells originating from all three germ layers, the cardiogenic lineage included [18]. The authors of the cited study claim that the phenomenon has been observed with > 25 different human embryonic stem cell lines and induced pluripotent

stem cell lines. The increase of the yield of differentiated cells was reported to be dramatic (from 25 % up to > 90 % for different cell lines), even in these that have been known to have low propensity to differentiation into derivatives of specific embryonic layers [147, 148].

It has been proposed that the presence of DMSO increases the competence for differentiation in undifferentiated cells by induction of a transient G1 phase arrest [18, 149]. Normally, cells of early mammalian embryos and embryonic stem cells cycle rapidly, with significantly shortened G1 phase of the cell cycle. The G1/S phase checkpoint is activated in the presence of DNA damage and is associated with decreasing the risk for transmitting mutations to the cell's progeny [150-152]. The strictness of the control checkpoint in G1 is relaxed in embryonic cells compared to the requirements of G1/S transition in somatic cells [153]. What is more, mammalian embryonic cells are universally sensitive to the presence of DNA damage, but different types of embryonic cells may respond differently to it [152]. In early mouse embryos and in mESC (their in vitro counterparts) the G1 checkpoint is virtually switched off, allowing the cells to proceed with DNA replication even in the presence of DNA damage. Upon differentiation, the G1 checkpoint in mouse cells is restored and in differentiating cells the routine pre-replication checks for the presence of DNA damage are carried out in full measure. In early human embryos and undifferentiated hESC the G1 checkpoint is operative, but the control is significantly relaxed. Again, the strictness of the checkpoint is fully restored after the cells had begun differentiation. Thus, cells in the mouse embryo that have sustained DNA damage are rerouted to the differentiation pathway, ensuring the survival of the embryo at the expense of introduction of mutations, whereas human embryonic cells still must check for DNA damage in G1 phase and repair it within the time limits of the abbreviated G1 phase in order to be allowed to replicate their DNA [154-156]. Embryonic cells that have been temporarily trapped in G1 phase must either embark on the differentiation pathway, or else revert to apoptosis. DMSO functions as a differentiation agents by activation of the major G1 checkpoint control protein pRB1 (retinoblastoma protein). pRB1 functions as an inhibitor of the G1-S phase progression by binding and inactivating the transcription factor E2F (E2F1), thereby repressing the transcription of essential S-phase genes [157, 158]. The active (E2F-binding) form of pRB1 is hypophosphorylated. In late G1, pRB1 is phosphorylated by activated cyclin-dependent kinases in order to release E2F and allow the cell to proceed to S phase of the cell cycle [159, 160]. Treatment of differentiating embryonic cells with DMSO resulted in 4-fold increase in the levels of hypophosphorylated retinoblastoma protein [18]. Thus, it is proposed that following DMSO treatment the cell cycle is arrested at the pre-replication G1/S checkpoint, which is a potent trigger of the differentiation programme.

DMSO is a commonly used cryoprotector in cell culturing, used at concentrations up to 10 %. There is always some leftover DMSO in cells that have been frozen and recently thawed. It is possible that at least part of the reported cased of spontaneously differentiating cells are related to stimulation of the differentiation pathways by the presence of DMSO in the nutrient medium.

Alpha-lipoic acid has been shown to promote DMSO-induced differentiation of mouse embryonic carcinoma cells into the cardiogenic lineage [161].

Ascorbic acid is a potent stimulator of the differentiation of ESC into cardiogenic fate [162]. Supplementation with ascorbic acid greatly increases the yield of cardiomyocytes from human iPSCs (> 30-fold) [163]. However, the authors of the latter study report that there was a 'window' during which the application of ascorbic acid was effective (within the timeframe of specification of cardiac progenitors).

Supplementation of differentiating cells with 1,25-vitamin-D3 was shown to promote differentiation along the cardiogenic lineage via negative modulation of the canonical Wnt signaling pathway and the formation of cardiomyotubes [164].

Other small-molecule supplements, such as 5-azacytidine, alone or in combination, may also increase the yield of cardiomyocytes from pluripotent cell lines [165-167]. Studies on murine embryonic carcinoma cells showed that treatment with 5-azacytidine resulted in an increase in the levels of acetylated histone H3; upregulation of the expression of cardio-specific gene markers and in development of cardiomyocyte-rich embryoid bodies [168]. However, type of starting cells matters, as 5-azacytidine showed much lower efficiency in transdifferentiation of mesenchymal stem cells from bone marrow along the cardiogenic lineage and was not at all efficient with mesenchymal cells from umbilical cord and cord blood [165].

6. Markers for differentiation into cardiomyocyte lineage

The presence of at least partly functional cardiomyocytes in a cell culture is easily distinguishable by a single obvious morphological sign, namely, robust spontaneous contraction. According to most protocols, it ought to be noticeable by the day 12 since the first day of induction towards differentiation.

There are markers (detectable at nucleic acids and/or protein level) that may reliably indicate onset of differentiation and the phase of differentiation along the mesodermal and the cardiogenic lineage. Undifferentiated human cells express at high level the pluripotency markers

OCT4, SOX2 and NANOG, the cell surface markers stage-specific antigen-4 (SSEA4) and TRA-1-60; and alkaline phosphatase [169-171]. The levels of expression of these markers decline rapidly once differentiation has begun. The levels of OCT4, SOX2 and NANOG normally decrease in differentiated cells compared to cells maintained in the undifferentiated state, although the expression of OCT4 may persist for some time after differentiation has begun. This is probably related to the fact that the 'pluripotency' protein OCT4 may also contribute to lineage commitment in differentiating cells. A > 50 % decrease in the levels of Oct4 (relative to the level of expression in cells maintained in the undifferentiated state) was shown to divert murine cells towards trophectodermal fate, whereas a 50 % increase in Oct4 expression resulted in differentiation towards mesodermal and endodermal fates [172]. Recently, a Tcf/Lef-Oct4 composite binding site was identified in the promoter of *Mesp1* (a cardiac mesoderm-specific gene), which was shown to be identical in sequence to the Sox2-Oct4 binding site [173]. The Tcf/Lef-Oct4 site was shown to mediate the activation of the *Mesp1* promoter by Oct4 and canonical Wnt signaling [173].

The cell surface marker stage-specific antigen-1 (SSEA1, LeX/SSEA1/CD15) is typically not expressed in hESC maintained in the undifferentiated state, but its expression is quickly upregulated in cells that have embarked on the differentiation route and may therefore be used as an early differentiation marker [98]. Two other surface markers that may be used to indicate early stages of mesodermal differentiation are N-CAM (neural cell adhesion molecule, CD56) and EpCAM (epithelial adhesion molecule, CD326) [98, 174]. The emergence of mesoderm may also be monitored using the transcription factors BRACHYURY, SNAIL, TBX6 and FOXH1 [175-177].

Cells committed to the cardiac mesoderm lineage may be conveniently identified using only four surface markers – ROR2 (receptor tyrosine kinase-like orphan receptor 2, an inhibitor of Wnt canonical signalling); CD13 (alanine aminopeptidase, an antigen commonly present on the surface of leukocytes); VEGFR (KDR) and platelet-derived growth factor receptor alpha (PDGFRα, a cell-surface receptor for PDGF - A, B, and C) [178-182]. Human cells that are advanced into cardiogenic pathway of differentiation express cardio-specific proteins (mainly transcription factors) such as MESP1 (one of the first markers indicative of cardiogenesis), TBX5, GATA4, MEF2C, NKX2.5, and ISL1 [182]. The expression of key cardiac transcription factors such as NKX2.5 and TBX5 ought to be detectable in the majority of the cells by the 4th day of differentiation [27].

Even the most effective methods for targeted differentiation into the cardiogenic lineage do not produce pure cardiomyocyte populations, but a mixture of cardiomyocytes and other cells, including undifferentiated cells. The latter may be a source of serious trouble in potential clinical applications, as transplanted cells with high proliferative potential may produce rapidly growing tumours. Thus, the cardiomyocyte-rich population obtained by targeted differentiation needs to be purified before use. Markers specific for cardiac tissue may be used for identification of differentiated cardiomyocytes such as cardiac troponin T and I, cardiac alpha-myosin heavy chain, atrial natriuretic factor, and the cardiac transcription factors GATA-4, NKX2.5, and MEF-2 [183, 184].

In 2011; after screening of over 200 surface molecules, it was found that cardiomyocytes derived from human ESC or human iPSC may be identified with high specificity by the surface expression of vascular cells adhesion molecule 1 protein (VCAM1) [185]. Signal regulatory protein1 alpha type (SIRPA, a surface marker usually expressed on myeloid cells) has also been identified as a specific marker for differentiation into the cardiogenic lineage [186]. A combination of VCAM1, SIRPA and CD34 has been proposed as a mini-panel for identification of discrete stages of cardiovascular cell lineage differentiation [187].

7. Potential advantages of using cultured cardiomyocytes derived by differentiation from pluripotent stem cells lines

7.1. Research on the effect of different agents and different therapeutic regimens on the contractile function of the heart

There are significant advantages to using cardiac cells grown by differentiation from pluripotent cells or by reprogramming/transdifferentiation from other types of cells in studies of the effect of different agents on the human heart. Studying the effect of any agent on cultured cells, of course, is not quite the same as studying it in a complex organism. It might be, however, the next best thing, when it comes to experiments on vital organs and systems. Experimentation with therapeutic regimens that affect the functioning of the heart of human subjects and human-like primates is not acceptable, except in cases when the safety of the experiment ***and*** the potential benefits for the patient were repeatedly demonstrated in previous trials on other model systems. Thanks to the achievements of modern research and molecular diagnostics it is now possible that even minute changes representing a response to drugs affecting the functioning of cultured cells may be detected in real time. Thus, after extrapolation, the potential effects of the agent on tissue and organ level may be anticipated with a reasonable level of certainty and without risk for causing irreversible

damage to patients or volunteers.

The supply of cardiomyocytes obtained by differentiation from ESC or iPSC is, at least in theory, virtually inexhaustible. Provided that the original cell line is managed in its undifferentiated state (preserving its replicative capacity as well as its differentiation potential) and that the differentiation is carried out according to the standardised protocols, the cells used for every experiment would be essentially the same as in previous experiments. Reproducibility (consistency) of results is a very important factor in research (for details, see below). Obtaining consistent results with the same cell line and between cell lines treated with the same agent or regimen increases the likelihood that the signals generated in response to the agent or the regimen were truly product of the experiment procedure and not artefacts brought by a fluctuation in the experimental conditions.

7.2. Animal and human biology may sometimes be very dissimilar

7.2.1. Animals do indeed make us human

Non-primate animals (mice and rats, rabbits, dogs, sheep, swine, and others) have been used for studying the functions of mammalian body for centuries, and, in the last several decades, for modelling human biology in physiological and pathological conditions. Even the most promising trials conducted on animals, however, may sometimes produce results that are not directly applicable (or may even be drastically different) from the results obtained when the same agent is used in human beings. It is sufficient to mention the infamous phase III clinical trial of the neuroprotector PBT that was shown to be very effective in prevention of spreading of brain damage after stroke in many animal species, but did not exhibit detectable activity when tested in human patients [188, 189].

However close non-primate mammalian species might be to man with respect to their DNA sequence and chromosome structure, they may not always constitute adequate models. Apparently, there may be small but very significant differences between the physiology, the metabolism and the molecular mechanisms of basic cellular processes between animals and man. For example, the cardiovascular system of swine is similar to the cardiovascular system in humans with regard to size and internal organisation of the heart, its pumping capacity and the pulse rate (slower than in most medium-sized animals and close to normal human heart rate) [reviewed in 190]. However, as much as porcine hearts look and function as human hearts, they offer, at best, an approximation. The shape of the heart and its position within the thorax are different between pigs and humans. Quadruped animals have the posterior heart surface adjacent to the diaphragm, and the anterior surface against the sternum. Acquisition of bipedal stance in the course of evolution placed additional strain on the heart and the internal organs, causing the human heart to 'ride' the diaphragm [190]. Such differences may matter significantly when it comes to cardiac reconstruction and heart transplantations. Porcine heart valves are now routinely used in human cardiac repair, but, at present, hearts from genetically engineered ('humanised') pigs have been transplanted in low primates (baboons) only, and with moderate success (the transplanted organs functioned for 6-8 months) [191, 192].

7.2.2. Disease-specific human stem cell lines get as close as possible to modelling human disease

The signals generated by a living model in response to external stimuli, even under strictly controlled experimental conditions, may be different in a human being affected with a certain disease or condition and in animal models of the same disease or condition. When modelling human disease in lower mammalian species, the associated signs and symptoms and the objective pathological changes may be different between humans and animals. This is specifically true when these changes affect the functioning of the central nervous system, but differences may surface in modelling disease of virtually all organs and systems in animals. In such cases, human and primate disease cell lines may be of great value, as they may provide direct insight into the molecular mechanisms underlying the disease process, meaning that the effects of the studied agents and therapeutic regimens may be observed without being masked or compensated for by other effects that occur in living organisms. This is as close as possible to modelling pathology of diseased human tissues and organs, albeit the effects of the integrative relationship at system and organism level may be lost in research in cultured cells. It is believed that disease cell lines may greatly facilitate drug development and accelerate the trials of various therapeutic agents, at the same time minimising the inherent risk of causing damage to human patients because of potential adverse effects.

The first human disease cell lines were derived from embryos with known genetic defects (usually, confirmed by preimplantation diagnostics and therefore deemed unfit for use for reproduction purposes). At present, much effort is invested into creating iPSC lines derived from reprogramming of somatic cells or progenitor cells (e.g. cells from umbilical cord or adult stem cells such as haematopoietic progenitors from bone marrow) from individuals with specific genetic backgrounds that may be associated with high risk for development of disease (patient-specific disease cell lines). Several years ago

the number of established human disease cell lines was in the order of several dozens, but the number of lines available for research has grown exponentially since [summarised in 193, 194]. Recently, it has been reported that patient-specific iPSC cardiomyocyte cell lines were established for common inherited cardiac arrhythmias - various subtypes of long QT (LQT) syndrome subtypes; the mixed phenotype of LQTS3/Brugada syndrome; subtypes 1 and 2 of catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular cardiomyopathy [23, 195].

7.2.3. The Methuselah of mice – modelling reliably common multifactorial diseases and conditions on a short-living animal model is hardly likely

There is a specific issue in the area of modelling human diseases and conditions that are associated with ageing, on animals. These are conditions that typically occur late in human life (as is cardiovascular disease); and/the occurrence of which is dependent on the accumulation of events over many years (as are normal and pathological ageing of tissues and organs and cancer). Most routinely used laboratory animals are much shorter-lived than man. Mice and rats rarely live over 3 years, even in optimal conditions. Domesticated dogs and cats, depending on the breed, may live for 10-15 years, rarely longer. The average life expectancy of sheep and swine is in the same order. In contrast, higher primates have an average life expectancy of 5-6 decades in captivity and humans of today are expected to live at least 80 years. Indeed, the life cycle programming of virtually all eukaryotes currently living on Earth includes ageing and 'death of old age', but the difference between animal and human life expectancies may make some of the characteristics of 'diseases of old age' very different between animals and humans. For example, 'diseases of old age' (cardiovascular disease, lung disease, glucose intolerance of the elderly, joint disease, senile cataract, etc.) in short-living animals may not be associated with simple 'wear and tear' of tissues and organs occurring after many years of use. The DNA sequence, the gene content, the structure of the genome; the organisation and the functioning of the tissues and the organs of the man and the most commonly used laboratory animal - the mouse - are remarkably similar to one another to explain why the one species lives 20-30-times longer than the other. It is likely that the crucial differences are at molecular level and, therefore, reflect on multiple aspects of animal and human biology.

Cardiovascular damage (related to cardiac hypoxia, ischemia, etc.) and cerebrovascular damage (e.g. in stroke) is accompanied with significant increase in the amount of oxidative DNA damage. In the vicinity of the lesion site, the rate of DNA repair and the rate of apoptotic cell death in cells that had sustained irreparable damage play crucial role in the development of pathological changes in the affected tissue and the subsequent recovery. Rodents are known for their unique pattern of DNA repair, targeting the repair efforts predominantly towards the transcribed regions of the genome, whereas damage in untranscribed regions may be left unrepaired (popularly called 'the rodent repairadox') [196, 197]. It allows cells to survive significant amount of DNA damage at the expense of increased risk for mutagenesis. In contrast, cells from primates (including humans) would attempt to repair all DNA damage (admittedly, starting with the transcribed regions of the genome) and are likely to switch on the apoptotic pathways if the damage has proven to be too extensive to be repaired, even if it is in untranscribed regions of the genome. Leftover DNA damage in eukaryotic cells capable of replicating their DNA is associated with significant increase in the risk for accelerated ageing and/or cancer. Mice and rats rarely live beyond 2-3 years and their cells reach replicative senescence only after 15-18 divisions [198]. Humans of today, however, are likely to live >80 years and the typical human cell divides over 50 times before replicative senescence settles in. It could easily be seen that a DNA repair system that would ignore the nontranscribed regions of the genome may suit perfectly the needs of a short-living animal, such as the mouse, but not much longer-lived living beings, such as humans. The persistence of DNA damage in division-competent cells would accelerate the ageing process, bringing on the 'diseases of old age' (cardiovascular disease, joint disease, cancer, and others). The latter are likely to significantly shorten the life expectancy below what's the average for a long-lived species. Thus, a small difference at molecular level (pattern of DNA repair between transcribed and untranscribed regions) may have much larger and diverse consequence for different living beings. These inter-species differences in biology of late life are likely to become greater with increasing difference between the average life expectancy of the two species. Apparently, it would be difficult to model long-term effects (normal ageing, 'diseases of old age', propensity for spontaneous tumorigenesis, etc.) on an animal that only lives for a couple of years.

Considering this, cultured human cells are likely to provide more valuable information than animal models. Cultured cardiomyocyte progenitors (cardiac progenitor cells, vascular stem cells) and cardiomyocytes derived from pluripotent cells could be maintained in vitro for years, and, potentially, for decades. Unlike differentiated adult cardiomyocytes that are replaced (albeit at a rate of 5-10 % per decade), cardiomyocyte progenitors were programmed to last throughout a normal human life, as they have been found in hearts of nonagenarians

[35]. Thus, cultured cardiac cells may be preferable to living animals as research models, despite the levels of integration and complexity that animals may offer. This is valid for research carried out in physiological conditions (e.g. normal ageing, effects of various agents on myocardial functioning, etc.) as well as for research involving treatment of disease, especially for modelling multifactorial diseases and conditions with significant genetic component that usually manifest in later life.

7.3. A matter of consistency - one cannot evaluate results of research adequately without standardising the conditions for derivation, maintenance and targeted differentiation of stem cell lines

Much effort has been invested in making work with stem cells reproducible – that is, making possible that independent research groups may obtain consistent results with the same cells by treating them with the same compounds and under the same conditions. At present, however, different stem cell lines still may show remarkably dissimilar properties with regard to differentiation into various types of specialised cells. This prompted some authors to introduce the term 'lineage scorecard', assessing the potential for differentiation of a stem cell line to specific lineages or specific cell types [148, 199]. There are human cell lines (e.g. HUES6) that are known to be poor performers when directed for differentiation into endodermal lineage and others (HUES8) that exhibit significantly higher propensity for differentiation into endoderma than other human stem cell lines [139, 147]. Even among these cell lines that score very high on the 'lineage scorecards' for a particular lineage, the percentage of specialised cells that are produced by targeted differentiation may significantly vary between experiments and between laboratories. Indeed, there have been recent developments in experimental techniques that were shown to increase the differentiation rates for almost all cell types [18], but the consistency of the differentiation rates into the cardiogenic lineage still leaves much to be desired, especially with embryoid bodies-based methodology for differentiation. The latter typically yields no more than 8-10 % of pulsating cardiomyocyte-like cells and about 1-2 % of true cardiomyocytes [13, 200, reviewed in 98]. Such yields render the methodology suitable only for some types of research purposes that require production of differentiated cells on a mini-scale.

The low yield of robustly beating cardiomyocytes was initially believed to be related to the use of compounds with undefined or varying composition (bovine fetal serum; animal hormones and growth factors, extracellular matrix secreted by animal cells (e.g. Matrigel) and other products of xenotic origin). Batch-to-batch variations have been reported in the specialised literature and selection of a 'batch that works' has been a standard instruction in the early days of stem cell culturing. Indeed, this might have been a part of the problem, but the use of products of animal origin becomes more and more limited in modern research and cells grown in completely cell-free conditions are often reported to exhibit the same unsatisfactory differentiation rates as the same cell lines grown in medium supplemented with animal serum [reviewed in 98]. Some of the poorly performing stem cell lines (e.g. HUES1) were later found to have some of their basic differentiation routes selectively suppressed, which could explain the low differentiation rates into particular lineages [148, 201].

Consistency in the quality of the product is crucially important in manufacture of medicinal products that are likely to be used in humans, including cell and tissue preparations [EudraLex - The rules governing medicinal products in the EU, vol. 4 - GMP Guidelines]. Use of fully defined nutrient media and support substrates only is specifically indicated for products that may potentially be used in human beings. This is related to safety concerns about the possibility of introduction of undesired proteins and DNA of animal origin in the cell preparation that may interfere with the properties of the cells or may cause adverse reactions. New techniques for extraction and propagation of stem cells in culture have been developed that exclude any products of xenotic origin [202-205], but virtually all of the initially established cell lines had been grown in the presence of heterologous protein and/or heterologous cells. Concerns about the quality of stem cell lines established in conditions that were not 'xeno-free' had already been raised by some authors [206-208]. The consolidated opinion in the field at present is that the establishment of stem cell lines ought to be started again from scratch. Thus, the differences in the yields (and, sometimes, the properties) of cardiomyocytes derived from pluripotent cell lines or from other cell types may be related to inconsistent experimental conditions, but it is more likely that many of the currently existing cell lines were derived and maintained for a long time in conditions which might have reflected on their properties (e.g. replicative capacity, differentiation potential, etc). Hopefully, standardisation of the conditions for xeno-free derivation, maintenance and differentiation of human stem cells and establishment of new cell lines that have been grown in completely defined conditions would, with time, increase the reliability and reproducibility of targeted differentiation of stem cells. The latter would facilitate the accumulation of critical mass of knowledge about properties of in vitro differentiated cells so that the development of research applications and the potential clinical uses are speeded up and the preparations from stem cells are made safer and better.

8. Conclusion

At present, there are several well-developed approaches for differentiation of pluripotent stem cells and reprogramming of other types of cells along the cardiomyocyte lineage - the spontaneous differentiation method (augmented by small molecules, such as DMSO), the Activin A/BMP4 method and the Wnt signalling modulation method. Components of the one methodology may be included in the other (e.g. GSK3 inhibitor pretreatment typical of the Wnt signalling modulation protocol may be included in the Activin A/BMP4 protocol). The yield of robustly beating cells expressing cardio-specific RNAs and proteins may vary from several per cent (usually, in embryoid bodies-based protocols) to 70-90 % when the basic induction protocols are complemented by supplementation with small molecules such as DMSO, ascorbic acid, 5-azacytidine, etc. or, in the case of for the Activin A/BMP4 protocols, with a second 1-day induction. Still, there are inconsistencies between the results obtained by the different research groups working with the same protocols but with different cell lines and sometimes between experiments carried out with the same cell line. Standardisation of protocols and introduction of defined and xeno-free conditions for derivation, maintenance and targeted differentiation of pluripotent and multipotent cells into functional cardiomyocytes would result in higher reproducibility of results between experiments and between laboratories. The opportunity for testing new drugs and new therapeutic regimens on differentiated human cells instead of animal models would allow for safer, more rapid and more precise analysis of the effects of a specific agent or a regimen potentially intended for human use. In the long-term, consistency in the quality of the stem cell lines used for production of differentiated cells for clinical purposes may assist in the development of a more effective system for donor-recipient matching, possibly resulting in higher success rate of clinical applications.

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